Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 2804–2808, April 1992
Cell Biology

Isolation of a candidate human hematopoietic stem-cell population

CHARLES M. BAUM*†, IRVING L. WEISSMAN*†, ANN S. TSUKAMOTO*, ANNE-MARIE BUCKLE*, AND BRUNO PEAULT*‡

*SyStemix Inc., 3400 West Bayshore Road, Palo Alto, CA 94303; and †Howard Hughes Institute, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, CA 94305

Contributed by Irving L. Weissman, November 4, 1991

ABSTRACT **We have identified a rare (0.05–0.1%) subset of human fetal bone marrow cells that contains multipotent hematopoietic precursors. The population of human precursor cells that express Thy-1 and CD34 but no known lineage markers is enriched for clonogenic activity that establishes long-term, multilineage (myelomonocytic and B lymphoid) cultures on mouse marrow stromal lines. Further, the Thy-1$^+$CD34$^+$ subset that takes up little of the fluorescent mitochondrial dye rhodamine 123 contains virtually all the cells that establish long-term cultures. In human fetal thymus transplanted into SCID (severe combined immunodeficiency) mice, Thy-1$^+$CD34$^+$ fetal bone marrow cells differentiate into T lymphocytes. In two of nine cases, allogeneic Thy-1$^+$CD34$^+$ cells could engraft intact human fetal bone marrow grown in SCID mice, resulting in donor-derived myeloid and B cells. By extrapolation, the rare human Thy-1$^+$Lin$^-$CD34$^+$ cell population contains pluripotent hematopoietic progenitors; we propose that it is highly enriched for candidate hematopoietic stem cells.**

In bone marrow (BM), the main blood-forming organ in the developed mammal, cascades of stem-cell divisions give rise to most hematolymphoid cell populations (1–3). Of these, only totipotent hematopoietic stem cells (tHSCs) can reconstitute lethally irradiated animals by giving rise to all blood cells, including progeny HSCs.

Isolation of candidate HSCs in the mouse required the development of assays for clonogenic precursors of the T [thymic colony-forming unit (CFU-T)], B, and myeloerythroid [splenic CFU (CFU-S)] lineages (4–6); such precursors lack detectable surface markers of the T, B, macrophage, granulocytic, and erythroid lineages [lineage-negative (Lin$^-$)] (7) but express the Sca-1/Ly-6A antigen and low levels of the Thy-1 molecule (7–9). This Thy-1^{lo}Lin$^-$Sca-1$^+$ population, representing ≈0.05% of mononucleated BM cells, is the only subset that initiates long-term BM stromal cultures; it is 1000- to 2000-fold enriched in the capacity to save lethally irradiated animals and reconstitute them long term with donor-derived cells in all hematolymphoid lineages (7, 9). Independent attempts to isolate tHSCs have utilized other cellular properties (10–13) and demonstrated their activity *in vitro* or *in vivo* (14–16). In some studies, long-term reconstitution activity was separable from radioprotective and CFU-S activity (17). For example, the Thy-1^{lo}Lin$^-$Sca-1$^+$Rh-123^{lo} subset (comprising cells that take up little of the mitochondrial dye rhodamine 123) is enriched for self-renewal and long-term reconstitutive potential compared with its Rh-123^{hi} counterpart (18).

Most attempts to identify human HSCs have utilized the high proliferative response of such cells *in vitro* in the presence of hematopoietic cytokines and have revealed CD34 to be a potent cell surface marker of such progenitors (19, 20); in mice, HSCs are not the only such cytokine-responsive cells (21). Several groups have developed human stromal cell-dependent long-term culture systems that have identified progenitors of the myeloerythroid type (22–24). The stromal culture system described here allows single human progenitor cells to differentiate into both the myeloerythroid and B-lymphoid lineages.

Experimental *in vivo* hematopoietic assays are not practicable with humans, although autologous CD34$^+$ cells can radioprotect primates (25) and promote hematopoietic recovery in humans (26). To assay human progenitor cells *in vivo*, we have used the SCID-hu model, wherein human blood-forming organs develop in the immunodeficient SCID mouse (27). In this study, SCID-hu and stromal culture assays have identified a rare (0.05–0.1%) human fetal BM Thy-1$^+$CD34$^+$ population that contains multipotent hematopoietic progenitor activity.

MATERIALS AND METHODS

Monoclonal Antibodies. mAbs were purchased from Becton Dickinson (CD3, -4, -7, -8, -10, -14, -15, -16, -19, -20, and -33) or from AMAC (CD35, -38, and -71). mAbs against HLA class I antigens were derived from hybridomas obtained from the American Type Culture Collection. mAbs to human CD34 (Tuk3) and to human Thy-1 (F15 421-5) were obtained from Andreas Ziegler (University of Berlin, Germany) and from John Fabre (Oxford University, England), respectively.

Mouse Stroma/Human Hematopoietic Cultures. Whitlock–Witte BM stromal cell lines were established from BALB/c mice (28, 29). Bulk or limit dilution cocultures were initiated using fetal or adult BM cells on confluent mouse stromal cells (C.M.B. and A.S.T., unpublished work).

Preparation of Human Hematopoietic Cells and Fluorescence-Activated Cell Sorting. Samples were prepared and sorted as described on a modified Becton Dickinson FACStar Plus (30, 31); cells stained with Rh-123 (18) were sorted into Rh-123^{hi} (30%) and Rh-123^{lo} (30%) subsets. Single cells were deposited using the automatic cell deposition unit module.

Colony Assays. The methylcellulose assay was performed with placental or Mo conditioned medium (8, 24).

***In Vivo* Stem-Cell Assays.** The transplantation of sorted cells into the human fetal thymus to be engrafted into SCID mice was performed as described (31). Human fetal bones (18–24 weeks) were sectioned into 4- to 6-mm cylindrical fragments, microinjected with cells in a volume of 1–3 μl, and transplanted into the peritoneal cavity of SCID mice. Repopulation of human fetal thymus and BM grafts by HLA-

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Abbreviations: BM, bone marrow; BFU-E, erythroid burst-forming unit(s); CFU, colony-forming unit(s); FACS, fluorescence-activated cell sorter; HSC, hematopoietic stem cell; tHSC, totipotent HSC; Rh-123, rhodamine 123.

†Present address: Monsanto, Immunology, 700 Chesterfield Village Parkway, St. Louis, MO 63198.
‡Present address: Institut d'Embryologie Cellulaire et Moleculaire du Centre National de la Recherche Scientifique, 49 bis, avenue de la Belle Gabrielle, 94736 Norgent-sur-Marne, Cedex, France.

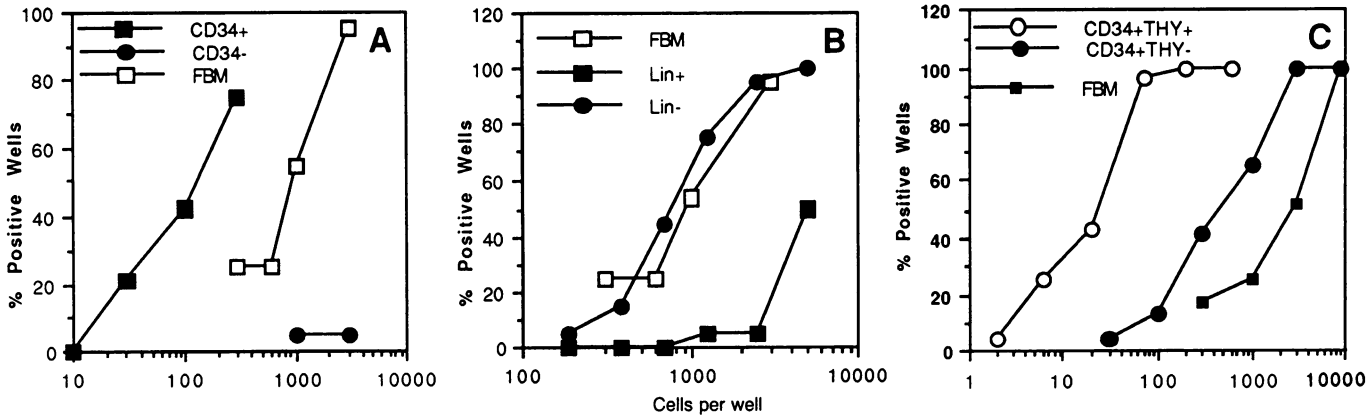

FIG. 1. Limit dilution analysis of fetal BM cell growth in long-term, stromal cell-dependent cultures. Fetal BM subpopulations were cultured in 96-well plates containing mouse BM stromal cells. Cells were titrated into the plates in serial dilutions with 24 wells at each dilution. Wells containing >1000 cells were counted as positive. Growth positive wells were counted at days 28–42. Frequencies were determined by the number of cells required to give 63% positive wells. In *A*, *B*, and *C*, fetal BM cells were sorted for CD34, Lin (CD3, -10, -14, -15, -19, -33) markers, and CD34 plus Thy-1, respectively.

disparate stem cells in SCID mice was evaluated as described (27, 31). Only experiments in which both whole BM and sorted populations yielded colonies in methylcellulose and/or coculture assays were included.

RESULTS

Human Fetal BM Subpopulations That Establish Long-Term Myeloid and Lymphoid Cultures. The addition of human hematopoietic precursor cells to a cloned murine BM stromal cell line allows the simultaneous differentiation of B-lymphoid and myeloerythroid lineages (C.M.B. and A.S.T., unpublished work). Limit dilution experiments showed that the BM cells that initiate these cultures were contained in the $CD34^+$ fraction (Fig. 1*A*; see also Table 2). However, the $CD34^+$ population was heterogeneous (refs. 32–35; Table 1); the majority of $CD34^+$ cells expressed markers of a mature blood-cell lineage. Cells lacking these markers (Lin^-) were significantly more effective than Lin^+ cells in establishing long-term cultures (Fig. 1*B*). Subfractionation of BM $CD34^+$ cells into the 5% $Thy\text{-}1^+$ and 95% $Thy\text{-}1^-$ subsets showed that the $Thy\text{-}1^+CD34^+$ cells initiated long-term cocultures at a significantly higher frequency than their $Thy\text{-}1^-CD34^+$ counterparts (Fig. 1*C* and Table 2).

To analyze the differentiation potential of single progenitor cells, a coculture assay was developed in which individual cells were deposited onto established stromal cells. The cultures were scored for wells containing >100 cells over a 4- to 6-week culture period to determine (*i*) the frequency of cells that initiate long-term cultures (>100 progeny) in the absence of any other human hematopoietic cells and (*ii*) the frequency of cells with the capacity to differentiate into both B-lymphoid ($CD10^+19^+$) and myeloid ($CD15^+33^+$) lineages (Tables 2 and 3). The majority of cells that established these long-term cultures resided in the $Thy\text{-}1^+CD34^+$, Lin^-CD34^+, and $Thy\text{-}1^+Lin^-$ cell populations (Table 2). In addition, the $Thy\text{-}1^+CD34^+$ population was highly enriched for multipotent cells that gave rise to both B lymphocytes and myeloid cells. In this assay, $Thy\text{-}1^+Lin^-CD34^+$ cells were enriched at least 10-fold over the $Thy\text{-}1^-Lin^-CD34^+$ cell population (Table 2). Further, cultures initiated by $Thy\text{-}1^+CD34^+$ cells could be replated, indicating that the cells capable of generating long-term cultures were present for up to at least 16 weeks (data not shown). Since the $Rh\text{-}123^{lo}$ subset of human BM has been reported to be enriched in primitive hematopoietic progenitors (36), the $Thy\text{-}1^+CD34^+$ cell fraction was

Table 1. Staining profile of fetal BM $CD34^+$ cells

Cell type	Marker	% stained*
T	CD3	0.8
	CD4	0.2
	CD8	0.1
	CD7	24
B	CD10	52
	CD19	53
Myeloid	CD14	2
	CD16	1
	CD33	32
Nonlineage	CD35	25
	CD38	95
	CD71	50
Unassigned	Thy-1	5

*Average of more than two experiments. Not all markers were stained in every case.

Table 2. Differentiation potential of various human fetal BM cell populations from limit dilution (*) or single-cell analysis

Population (% total)	Frequency of wells: Growth-positive	Frequency of wells: % with B and myeloid cells
Whole BM (100%) *	>1/3000	ND
$CD34^+$ (2–10%) *	1/200	ND
$CD34^-$ (90–98%) *	>1/50,000	ND
$Thy\text{-}1^+CD34^+$ (0.1–0.5%)	1/20	66
$Thy\text{-}1^-CD34^+$ (2–10%)	1/323	0
$Thy\text{-}1^+Lin^-$ (0.1–0.5%)	1/21	60
$Thy\text{-}1^+Lin^+$ (0.3–2%)	0/288	0
Lin^-CD34^+ (0.1–0.5%)	1/27	47
Lin^+CD34^+ (2–10%)	1/80	25
$Thy\text{-}1^+CD34^+Rh\text{-}123^{lo}$ (0.03–0.15%)	1/25	ND
$Thy\text{-}1^+CD34^+Rh\text{-}123^{hi}$ (0.03–0.15%)	0/1483	ND
$Thy\text{-}1^+Lin^-CD34^+$ (0.05–0.1%)	1/44	ND
$Thy\text{-}1^-Lin^-CD34^+$ (0.1–0.4%)	1/768	ND

Values represent the mean of two to six experiments. ND, not done. Pairwise combinations (e.g., $CD34^+/CD34^-$) were done in the same experiments. However, the $Rh\text{-}123^{hi}$ and $Rh\text{-}123^{lo}$ as well as the $Thy\text{-}1^+Lin^-CD34^+$ and $Thy\text{-}1^-Lin^-CD34^+$ populations underwent two rounds of sorting rather than one. Lineage markers included CD2, -3, -4, -7, -8, -10, -13, -14, -15, -16, -19, -20, and -33, but not all sorts had identical combinations of the above.

Table 3. Characteristics of cells derived from single Thy-1$^-$CD34$^+$ or Thy-1$^+$CD34$^+$ cells in coculture

	Thy-1$^-$CD34$^+$	Thy-1$^+$CD34$^+$	
Exp.	Frequency of growth-positive wells	Frequency of growth-positive wells	Frequency of mixed lymphoid/myeloid wells
1	0/384	1/18 (32/576)	1/1.3 (8/10)
2	1/384	1/35 (22/768)	1/1.5 (10/15)
3	0/96	1/56 (12/672	1/4 (2/8)
4	ND	1/12 (47/576)	1/4.3 (9/39)
5	1/155 (3/466)	1/15 (32/480)	1/1.3 (15/19)
6	1/288	1/14 (20/288)	1/1.8 (6/11)
Average	1/323 (5/1618)	1/20 (165/3360)	1/2 (50/102)

Numbers in parentheses indicate the total number of growth-positive wells (those with >100 cells) or mixed lymphoid/myeloid wells divided by the total number of wells analyzed in the experiment. ND, not done.

subdivided into Rh-123^{lo} and Rh-123^{hi} populations; preliminary experiments show that virtually all of the cells capable of initiating long-term cultures resided in the Rh-123^{lo} subset (Table 2). These data indicate that human pluripotent HSCs capable of initiating clonogenic long-term BM cultures are highly enriched in the Thy-1$^+$, Lin$^-$, CD34$^+$, and Rh-123^{lo} populations.

The Long-term Culture Cells Can Be Distinguished from Multilineage CFU-C (CFU-Cell). FACS-sorted fractions of fetal BM were placed into methylcellulose assays in the presence of cytokines and, in parallel, into the long-term coculture assay. Several FACS-sorted BM subsets responded to cytokines by developing colonies in methylcellulose, yet some of the same subsets (Thy-1$^-$CD34$^+$, Thy-1$^+$Lin$^+$) did not establish long-term cocultures (Table 4). As noted by others (22), cells that can initiate long-term cultures may score poorly in the methylcellulose assay. Preliminary analysis of the Thy-1$^+$Lin$^-$CD34$^+$ subset indicates that it has poor CFU-C activity under standard methylcellulose conditions. However, progeny of single cells from long-term cocultures that produced both B and myeloid cell progeny were also analyzed for CFU-C: in 11 of 13 cases, individual cells of the Thy-1$^+$CD34$^+$ phenotype gave rise to as many as 950 erythroid burst-forming units (BFU-E) and 850 CFU-GM after 6–8 weeks in coculture.

The Thy-1$^+$CD34$^+$ Cell Population Contains Thymic Lymphocyte Progenitors. The identification of mouse hematopoietic progenitors that differentiate to T lymphocytes relies on *in vivo* thymic reconstitution assays (6, 7, 37–39). HLA-marked CD34$^+$ cells microinjected into human fetal thymus just prior to its implantation into SCID mice yield progeny T lymphocytes (31). Here we show that Thy-1$^+$CD34$^+$ cells can reconstitute human thymus in SCID-hu mice (Fig. 2): following a single injection of Thy-1$^+$CD34$^+$ precursor cells into the thymus, 11 of 16 thymic grafts observed 4–6 weeks later were chimeric, containing donor-derived T cells, with several long-term (>2 months) reconstitutions (Fig. 3). Thymic reconstitution was much less effective with Thy-1$^-$CD34$^+$ cells; at 4–6 weeks, 5 of 16 grafts were positive, and only one low-level long-term reconstitution was observed (Fig. 3). Whether this single reconstitution was due to rare contaminating Thy-1$^+$ cells or whether the Thy-1$^-$CD34$^+$ population included a progenitor is unclear. Thymic engraftment by donor BM cells in the SCID-hu mouse does not occur consistently (40).

Thy-1$^+$CD34$^+$ Fetal Precursors Can Engraft and Differentiate in Human Fetal BM in SCID-hu Mice. Human fetal bone grafts may become vascularized in SCID mice. Further,

Table 4. Differentiation potential of fetal human BM progenitor populations

Population (% total)	Coculture colony frequency	Methyl cellulose CFU-GM frequency
Whole BM (100)	>1/3000	1/5000
CD34$^+$ (2–10)	1/200	1/500
CD34$^-$ (90–98)	>1/50,000	>1/50,000
Thy-1$^+$CD34$^+$ (0.1–0.5)	1/20	1/570
Thy-1$^-$CD34$^+$ (2–10)	1/323	1/250
Thy-1$^+$Lin$^-$ (0.1–0.5)	1/21	1/900
Thy-1$^+$Lin$^+$ (0.3–2.0)	0/288	1/900
Thy-1$^+$CD34$^+$Rh-123^{lo} (0.03–0.15)	1/25	ND
Thy-1$^+$CD34$^+$Rh-123^{hi} (0.03–0.15)	0/1438	ND
Thy-1$^+$Lin$^-$CD34$^+$ (0.05–0.1)	1/44	ND
Thy-1$^-$Lin$^-$CD34$^+$ (0.1–0.4)	1/768	ND

CFU-GM, granulocyte/macrophage CFU; ND, not done. See Table 2 legend. Coculture frequency was defined in Table 2. Methylcellulose data are from cumulative experiments.

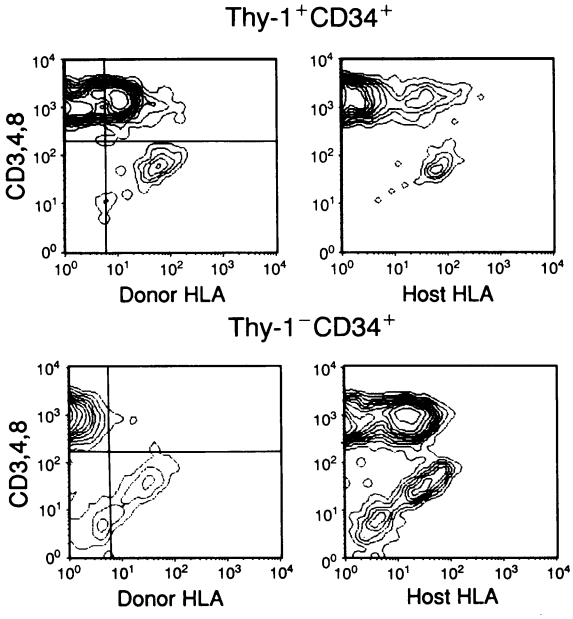

FIG. 2. Intrathymic T-cell development of allogeneic Thy-1$^+$CD34$^+$ fetal precursor cells. Human fetal thymus lobules (20 weeks of gestation) were cultured at 25°C for 7 days, microinjected with 2500 donor HLA-dissimilar Thy-1$^+$CD34$^+$ (*Upper*) or Thy-1$^-$CD34$^+$ (*Lower*) cells from fetal BM and then grafted under the kidney capsule of SCID mice for 9 or 11 weeks, respectively. Graft cells were analyzed on the FACS. The percentage of T cells expressing detectable levels of donor-specific HLA class I antigens was recorded (50% in the experiment shown here).

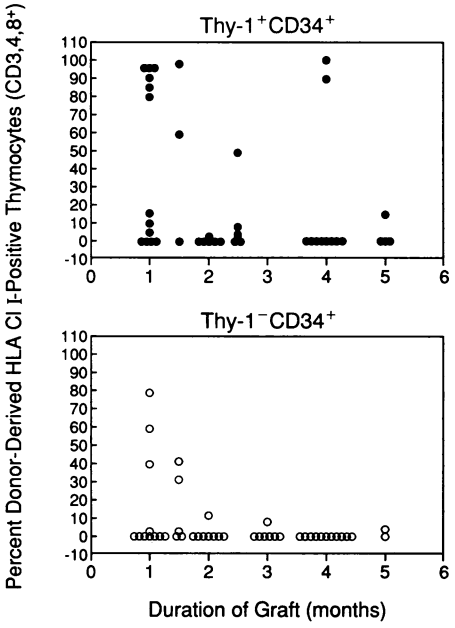

FIG. 3. Allogeneic T-cell repopulation of the SCID-hu fetal thymus by $CD34^+$ subsets with time. Donor-derived HLA class (CI) I-positive lymphocytes were quantified in human fetal thymus grafts colonized *in vitro* with 2.5–10 × 10^3 HLA-mismatched cells as described in Fig. 2. Data were collected from five distinct experiments where precursor cell engraftment and differentiation were observed.

FACS analysis of transplanted marrow reveals that human hematopoiesis in the myeloid ($CD33^+$) and B-lymphoid ($CD19^+20^+$) cell compartments can be sustained for 4–5 months (ref. 41, B.P. and C.M.B., unpublished observations). Fetal BM Thy-1^+CD34^+ and Thy-1^-CD34^+ cells were microinjected into the cavity of long bone segments and subsequently implanted into SCID mice. Failure to engraft in these unirradiated bones was often observed for HLA-mismatched donor/host combinations, regardless of the sorted cell population that was transferred. However, significant chimerism occurred in two of nine experiments as

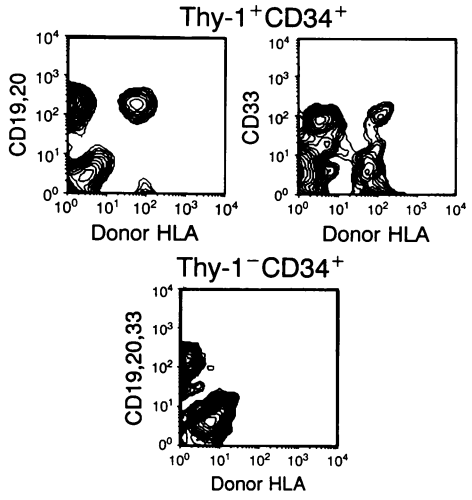

FIG. 4. Hematopoietic differentiation of allogeneic fetal Thy-1^+CD34^+ precursor cells in human fetal BM. The medullary cavity of long bone sections (22 weeks of gestation) was colonized *in vitro* by microinjection of 10^4 HLA-mismatched Thy-1^+ (*Upper*) or Thy-1^- (*Lower*) $CD34^+$ cells sorted from 20-week fetal BM, grafted intraperitoneally for 2 months in SCID mice, and then analyzed by FACS for donor-derived cells in the myeloid and B-lymphoid lineages.

Table 5. Percentage of donor-derived hematopoietic cells in human fetal bone segments injected with 10^4 donor cells

	2 months		3 months	
Population	% myeloid	% B	% myeloid	% B
Thy-1^+CD34^+	36	47	45	86
	13	20	6	1
	19	25	0	0
	ND	6	21	29
Thy-1^-CD34^+	0	0	0	0
	0	0	1	0
	0	0	0	0

Percentages of myeloid and B cells were determined 2 and 3 months after engraftment. Values represent percentage of donor B or myeloid cells from individual bone fragments from one experiment.

shown by the presence of a population of hematopoietic cells expressing donor-specific HLA class I antigens (Fig. 4). Donor-derived cells belonged to both the myeloid and the B-cell lineage (Table 5). Donor cell engraftment and maturation were observed following the transfer of Thy-1^+CD34^+ cells; the Thy-1^-CD34^+ population was consistently negative in the same experiments (Fig. 4; Table 5).

DISCUSSION

We have identified a rare set of Thy-1^+CD34^+ human fetal BM cells that is highly enriched for pluripotent progenitor activities in the myeloerythroid, the B-lymphoid, and the T-lymphoid system and for the ability to initiate long-term cultures. Since other subsets ($CD34^-$, Thy-1^-, or Lin^+) are relatively depleted of multipotent progenitors, Thy-$1^+Lin^-CD34^+$ cells should include the candidate human HSCs.

The identification of a HSC depends on one's definition as well as on the assays that subserve that definition. The most widely used test for human hematopoietic progenitors has been the methylcellulose assay for CFU-C and BFU-E activity (19, 20). Many non-stem-cell BM progenitors have been shown to be clonogenic precursors of these various colonies (21); therefore such *in vitro* colony assays may not be reliable indicators of the identity of the HSC.

We have used a long-term, stromal cell-dependent culture system that allows differentiation of myeloid and B-lineage cells (C.M.B. and A.S.T., unpublished work). In this system multipotent Thy-1^+, or Lin^-, or $CD34^+$ cells establish and maintain cultures. Furthermore, single cells of this phenotype reproducibly generate progeny of both the myeloid and B lineages. Moreover, individual Thy-1^+CD34^+ cells undergo amplification to produce large numbers of BFU-E and CFU-GM. Others have used a human Dexter culture assay to identify subsets of $CD34^+$ cells that initiate myelopoietic long-term stromal cultures (23, 24). The ability of these $CD34^+$ subsets to give rise to B lymphocytes is unknown. Here we show that the Thy-1^+CD34^+, Thy-$1^+Lin^-CD34^+$, and Thy-$1^+CD34^+Rh\text{-}123^{lo}$ subsets are highly enriched for cells that establish long-term cocultures.

We previously demonstrated that only $CD34^+$ progenitors could give rise to donor-derived reconstitution of human thymus (31); we have now demonstrated that the Thy-1^+CD34^+ subset has this activity. Similarly, in preliminary experiments, Thy-1^+CD34^+ HLA-marked donor cells injected into human fetal bones (thence placed into SCID mice) yielded donor myeloerythroid and B lineages. This assay is difficult: donor cells to be tested are in competition with endogenous human stem cells. In both the BM and thymic reconstitution experiments, detection of HLA-mismatched donor/host combinations was required. There are at least two major barriers to the transplantation of major histocom-

patibility complex (MHC)-incompatible hematopoietic cells in mouse and man; (*i*) the presence of MHC determinants that lead to predominantly T-cell-mediated rejection of transplants and (*ii*) a radiation-resistant barrier to MHC-incompatible hematopoietic cell transplants (42–45). Therefore, the use of MHC-incompatible SCID-hu mice for the assays of human progenitors may result in barriers to the transplantation.

Of course, the critical assay for human HSCs is human BM transplantation. The $CD34^+$ subset contains primitive progenitor activity *in vitro* (19–24) and *in vivo* (25, 26). The majority of $CD34^+$ cells are lineage-committed, and many leukemias express CD34 (20, 35), whereas expression of Thy-1 in leukemias has not been reported. Because only a rare subset of $CD34^+$ cells is simultaneously Thy-1^+, CD34 is not an efficient or exclusive identifier of HSCs.

The isolation of a candidate population of tHSCs should enable several important questions of clinical significance to be resolved. For example, it will be important to test whether candidate HSC populations are included in malignancies such as chronic myelogenous leukemia and whether human immunodeficiency virus genomes are detectable in this cell subset in AIDS patients.

We thank Drs. S. Heimfeld, G. Spangrude, and Y. Aihara for helpful advice and discussion. The expert technical support of N. Mori, A. Mizerek-Erhart, C. Garneau, S. Smith, and L. Osborne is acknowledged. Special thanks are owed to Dennis Sasaki for expertise and consultation in FACS analysis and to Edwin Yee and the Systemix animal facility and tissue procurement staff for providing SCID mice and fetal tissue. We thank K. Cranias and M. Finney for manuscript preparation. This investigation was supported by SyStemix, Inc. Development of the growth of hematopoietic cells *in vitro* was supported in part by Public Health Service Grant CD49605 awarded by the National Cancer Institute.

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